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Citation: Finn, Robert, McLaughlin, Lesley, Ronseaux, Sebastien, Rosewell, Ian, Houston, J. Brian, Henderson, Colin and Wolf, Roland (2008) Defining the in vivo role for cytochrome b5 in cytochrome P450 Function through the conditional hepatic deletion of microsomal cytochrome b5. The Journal of Biological Chemistry, 283. pp. 31385-31393. ISSN 0021-9258

Published by: American Society for Biochemistry and Molecular Biology

URL: <http://dx.doi.org/10.1074/jbc.M803496200>
<<http://dx.doi.org/10.1074/jbc.M803496200>>

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Supplemental Data

Defining the *in vivo* role for cytochrome *b*₅ in cytochrome P450 function through the conditional hepatic deletion of cytochrome *b*₅

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Supplemental Experimental Procedures

HPLC separation for microsomal incubations

HPLC analysis was carried out using a Hewlett Packard 1100 HPLC with UV detection and Chemstation software following the previously described conditions: bufuralol (1), chlorzoxazone (2). Metabolites were quantified with reference to authentic metabolite standards.

LC/MS-MS conditions for microsomal incubations

Microsomal product formation from phenacetin, midazolam, metoprolol and tolbutamide was analyzed by LC-MS/MS (Waters 2795 HPLC and Quattro Micro mass spectrometry system). The capillary temperature and voltage were respectively set at 250°C, 3 kV in positive and 250°C, 4 kV in negative electrospray ionization mode. Multiple reaction monitoring (MRM) data were acquired (Supplementary Table 1). Substrates, phenacetin, metoprolol and tolbutamide, were diverted in the waste. The cone

voltage and collision energy were optimized for each product (Supplementary Table 1); a dwell time of 0.5 s was used between MRM transitions.

4-Hydroxy midazolam, 1'-hydroxy midazolam and the internal standard (IS) phenacetin were resolved in 5 min on a Luna C18 (2) (5 μ , 100 Å, 50 x 2.00 mm) column (Phenomenex, Torrance, CA). The injection volume was 20 μ l. The following elution program was used at a temperature of 30°C and a flow rate of 0.5 ml/min: Eluent A – 0.1% formic acid; Eluent B – Acetonitrile; (i) a linear gradient from 3% to 50% B was run in 3 min, (ii) mobile phase was held at 50% B for 0.5 min, (iii) linear gradient was run to 60% B in 0.5 min, (iv) solvent composition was returned to 3% B for equilibration.

Acetaminophen and the internal standard caffeine were resolved with a Luna C18 (5 μ , 100 Å, 50 x 2.00 mm) column (Phenomenex, Torrance, CA) with a run time of 5 min. The injection volume was 5 μ l. The following elution program was used at a temperature of 20°C and a flow rate of 0.2 ml/min: 75% Eluent A – 20mM ammonium formic pH 2.6 and 25% Eluent B – Acetonitrile.

O-Desmethyl metoprolol, α -hydroxy metoprolol, metoprolol acid and the internal standard caffeine were analyzed with a Gemini C18 (5 μ , 110 Å, 50 x 2.00 mm) column (Phenomenex, Torrance, CA). The injection volume was 20 μ l. The following elution program was used at a temperature of 20°C and a flow rate of 0.3 ml/min: Eluent A – 10 mM ammonium formate pH 3.0; Eluent B – Acetonitrile with 0.1 % formic acid; (i) mobile phase was held at 9% B for 2.5 min, (ii) a linear gradient from up to 95% B was run in 1 min, (iii) mobile phase was held at 95% B for 2.5 min, (iv) solvent composition was returned to 9% B for equilibration.

4-Hydroxy tolbutamide and the internal standard chlorzoxazone were resolved using a Hypersil C18 (3 μ , 120 Å, 50 x 2.00 mm) column (Phenomenex, Torrance, CA). The injection volume was 50 μ l. The following elution program was used at a temperature of 20°C and a flow rate of 0.25 ml/min: 55% Eluent A – 20mM ammonium formic pH 2.6 and 45% Eluent B – Methanol.

LC/MS-MS conditions for blood samples

Blood extracts were analyzed for chlorzoxazone, metoprolol, midazolam, phenacetin, tolbutamide and the two internal standards; caffeine and reserpine. LC-MS/MS was carried out using a Waters 2795 HPLC coupled to a Quattro Micro Mass spectrometry system (MicroMass, Manchester, United Kingdom) in the positive and negative electrospray ionization mode. The capillary temperature and voltage were set as outlined above for the microsomal samples. Multiple reaction monitoring (MRM) data were acquired for each substrate (Supplementary Table 2). The cone voltage and collision energy was optimized for each compound (Supplementary Table 2); a dwell time of 0.1 s was used between MRM transitions.

Chromatography was carried out on a Luna C18 (2) (5 μ , 100A, 50 x 2.00mm) column (Phenomenex, Torrance, CA), with an injection volume of 10 μ l. The following elution program was used at a temperature of 20°C and a flow rate of 0.4 ml/min: Eluent A – 20mM ammonium formic pH 2.6; Eluent B – Methanol; (i) mobile phase was held at 15% B for 1 min, (ii) linear gradient was run to 75% B in 1 min, (iii) linear gradient was run to 90% B in 2 min, (iv) linear gradient was run to 95% in 0.5 min, (v) solvent composition was returned to 15% B for equilibration.

Reduced CO-difference spectra

P450 content of mouse liver microsomes was determined by P450 (Fe²⁺) - CO versus P450 (Fe²⁺) difference spectra from a sample of three pooled mouse livers as previously described (4).

Recapitulation of P450 activity method

HBN liver microsomes (25µg) were mixed with increasing amounts of *E.coli* membranes containing recombinant murine cytochrome *b*₅ (0-46 pmol of cytochrome *b*₅). Eppendorf tubes were capped and incubated at room temperature for 20 min. 190 µl of 100mM potassium phosphate buffer pH7.4 containing 30 µM BFC or 500 µM chlorzoxazone was then added and tubes mixed before placing into the wells of a 96 well plate. Reactions were initiated with 10 µl of 10 mM NADPH and run for 3 min (BFC) or 15 min (chlorzoxazone) at 37°C and metabolites measured as previously described. Cytochrome *b*₅ only and wild-type controls were also included.

Lipid Analysis

Detailed fatty acid profile analysis was carried out on liver microsomes, prepared as outlined in Experimental procedures, by the Nutrition Analytical Service, University of Stirling, UK.

For histology, liver samples were snap-frozen in Cryo-M-Bed (Bright Instrument Co, Huntington, UK), cryo-sectioned (10µM) and stained with Oil Red O (lipid content).

Supplemental References:

1. Paine, M. J., McLaughlin, L. A., Flanagan, J. U., Kemp, C. A., Sutcliffe, M. J., Roberts, G. C., and Wolf, C. R. (2003) *J. Biol. Chem.* **278**, 4021-4027
2. Peter, R., Bocker, R., Beaune, P. H., Iwasaki, M., Guengerich, F. P., and Yang, C. S. (1990) *Chem. Res. Toxicol.* **3**, 566-573
3. Flanagan, J. U., McLaughlin, L. A., Paine, M. J., Sutcliffe, M. J., Roberts, G. C., and Wolf, C. R. (2003) *Biochem. J.* **370**, 921-926
4. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370-2378

Supplemental Figure Legends

Supplemental Figure 1: Generation of hepatic cytochrome b_5 null mice. (A) Diagrams of the wild-type, targeted (floxed) and disrupted *Cytb₅* alleles. LoxP sites are indicated by triangles and the selectable marker (*hsv-tk-neo*) is indicated by a shaded box. The hatched box indicates the position of the probe used in Southern screening, and the numbered arrows indicate the position of the PCR primers used for screening, as detailed in the Experimental procedures. The dotted lines under the targeted (floxed) allele indicate the extent of DNA excision by Cre recombinase between the outermost loxP sites. (B) (i) Southern blot and (ii) PCR analysis of genomic DNA from *Cytb₅^{lox/+}*, *Cytb₅^{lox/lox}* and *Cytb₅^{+/+}* mice. For Southern blotting, the wild-type allele is represented by a band at 15 kb and the targeted (floxed) allele (*Cytb₅^{lox}*) containing the selectable marker by a band at 11 kb. *Note:* the targeted allele arises from Ola 129 genomic DNA, and the band generated by restriction digest on a Southern blot is smaller due to the presence of an additional *BglII* site in intron 3, which is not present in the gene locus in the C57BL/6 genome. For PCR analysis, the wild-type allele is represented by a band at 500 bp and the targeted allele (*Cytb₅^{lox}*) containing the selectable marker by a band at 900 bp.

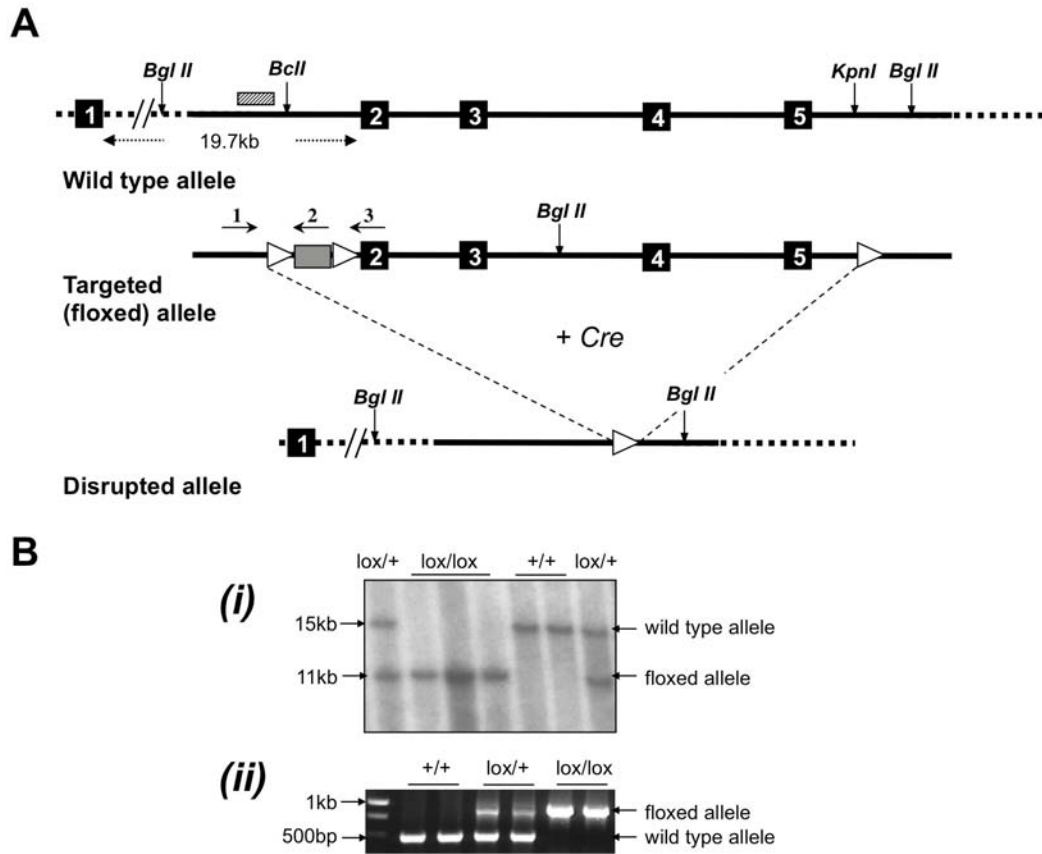
Supplemental Figure 2: (A) Reduced CO-difference spectra for HBN and wild-type (WT) liver microsomes and (B), (C) recapitulation of NADPH-mediated P450 activity towards BFC and chlorzoxazone, respectively, by supplementation of microsomes with exogenous cytochrome b_5 (11.5, 23, 34.5 and 46 pmol respectively) carried out as described in Supplemental Experimental procedures. Data is mean \pm SD.

Supplemental Figure 3: Lipid Analysis of HBN liver and microsomal membranes. (A) Hepatic lipid content was visualized by Oil red O staining of snap-frozen sections. Photomicrographs were taken at 10x magnification, bright field, and are representative of the results from at least three mice per group. (B) Fatty acid profile analysis of microsomal membranes (i) total lipid, (ii) saturated fatty acids and (iii) monounsaturated fatty acids. Black and white bars represent wild-type and HBN mice respectively. Data is expressed as a fold change compared to wild-type membranes.

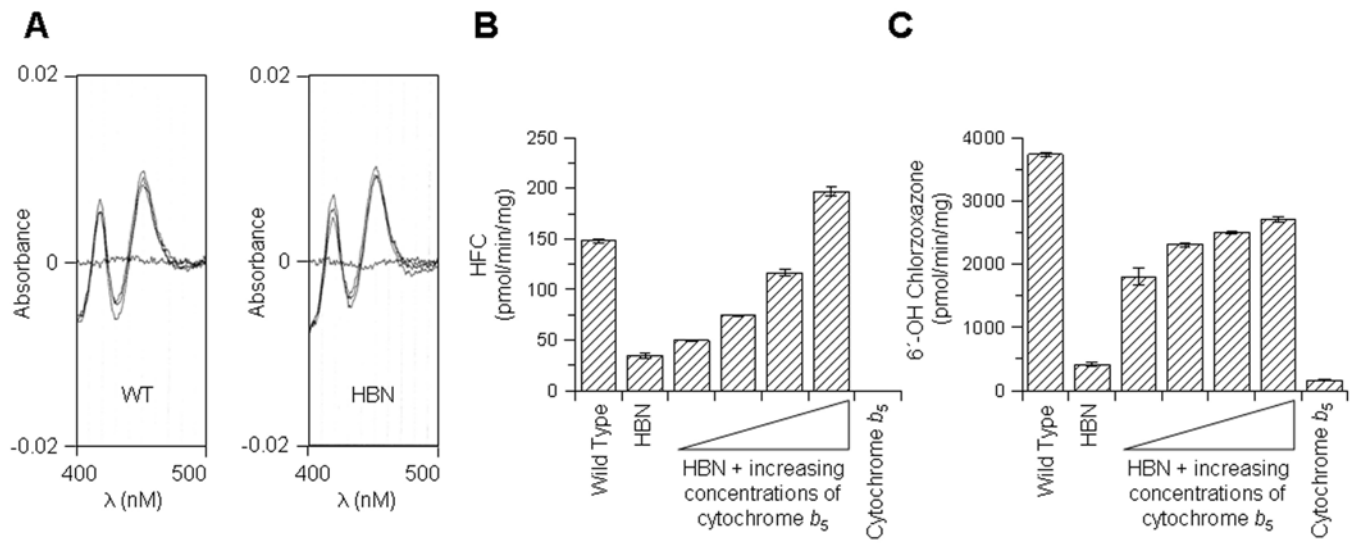
Supplemental Figure 4: Pharmacokinetic disposition of an orally administered P450 drug cocktail in wild-type and hepatic microsomal cytochrome *b*₅ null mice. A P450 drug cocktail containing chlorzoxazone (5mg/kg), metoprolol (2mg/kg), midazolam (5mg/kg), phenacetin (5mg/kg) and tolbutamide (5mg/kg) was administered orally to wild-type (black circles) or HBN (open circles) mice and blood samples taken at timed intervals to determine the *in vivo* pharmacokinetic parameters of the parent compounds. * = $p \leq 0.05$, ** = $p \leq 0.005$, *** = $p \leq 0.001$ where $n = 3$. Insert A shows pharmacokinetic disposition of orally administered tolbutamide (5mg/kg) as a single agent to wild-type (black circles) or HBN (open circles) mice ($n=2$ wild-type and 4 HBN).

Supplemental Figure 5: Measurement of metabolites for phenacetin (acetaminophen), metoprolol (O-desmethyl metoprolol) and midazolam (1'-hydroxy midazolam) from HBN (open circles) and wild-type (black circles) mice after intravenous (A) or oral (B) administration.

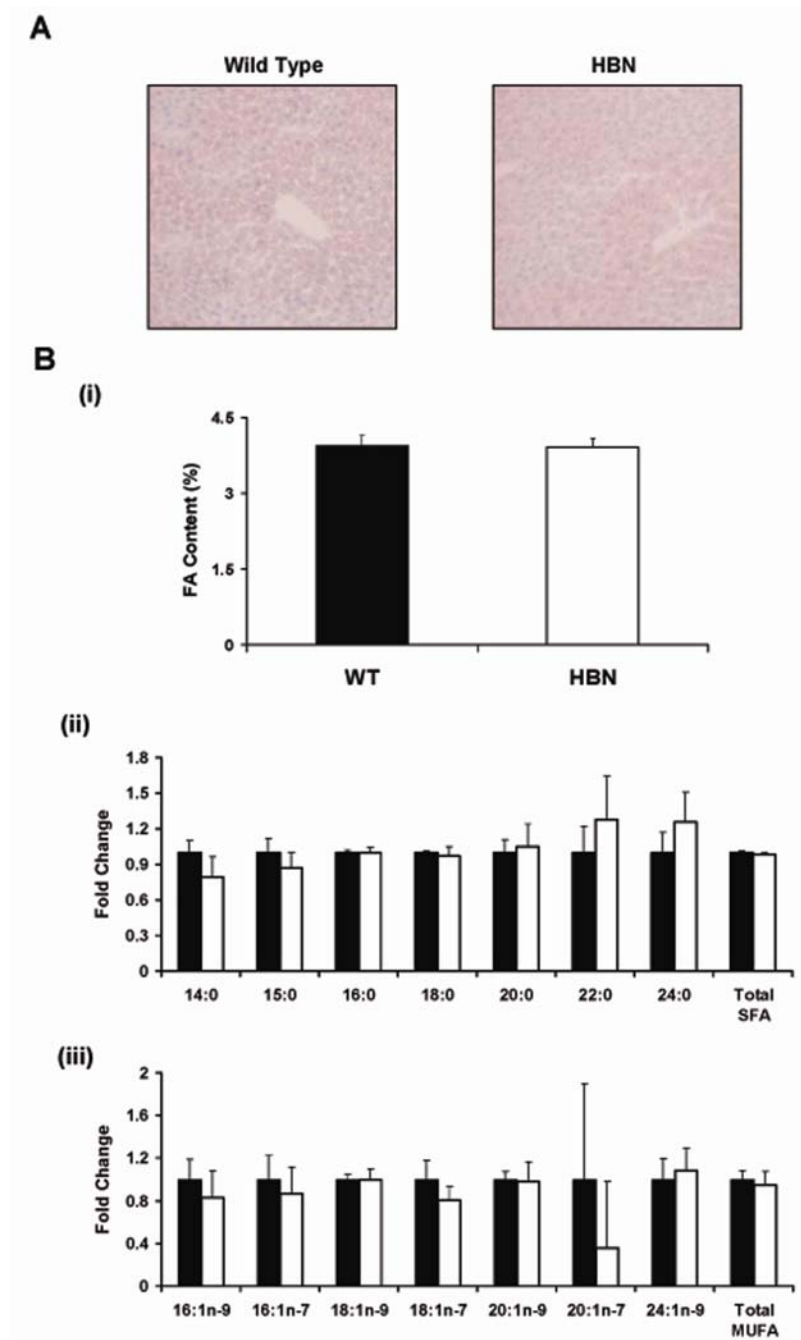
Supplementary Figure 1



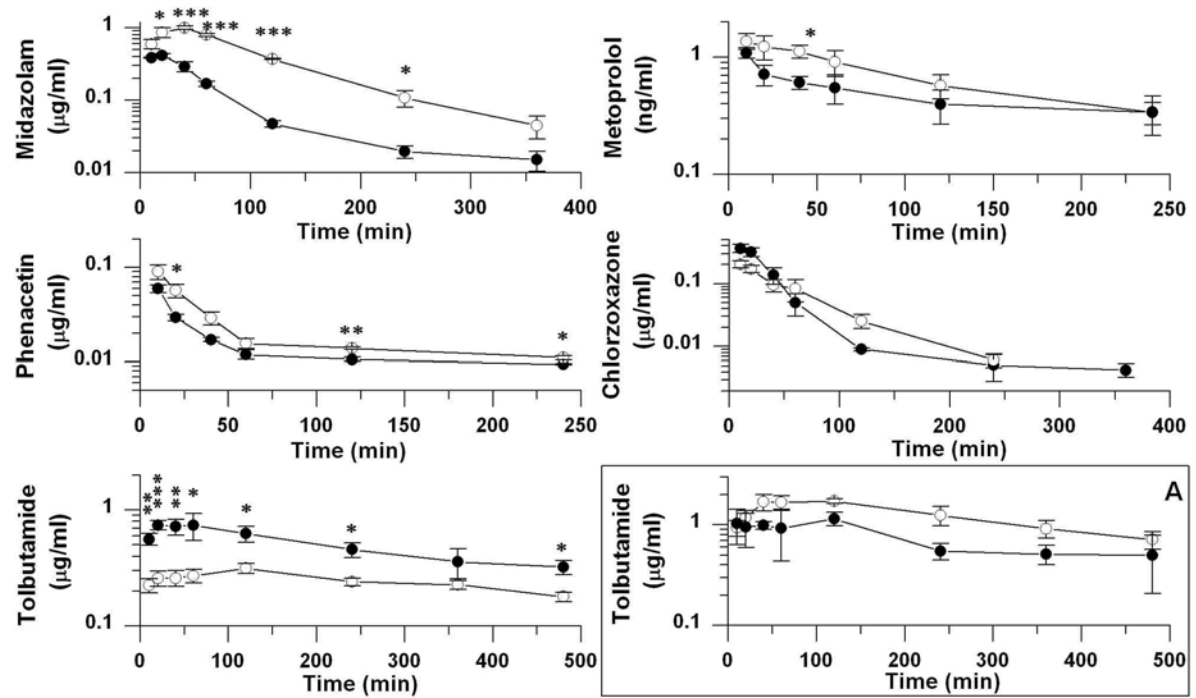
Supplementary Figure 2



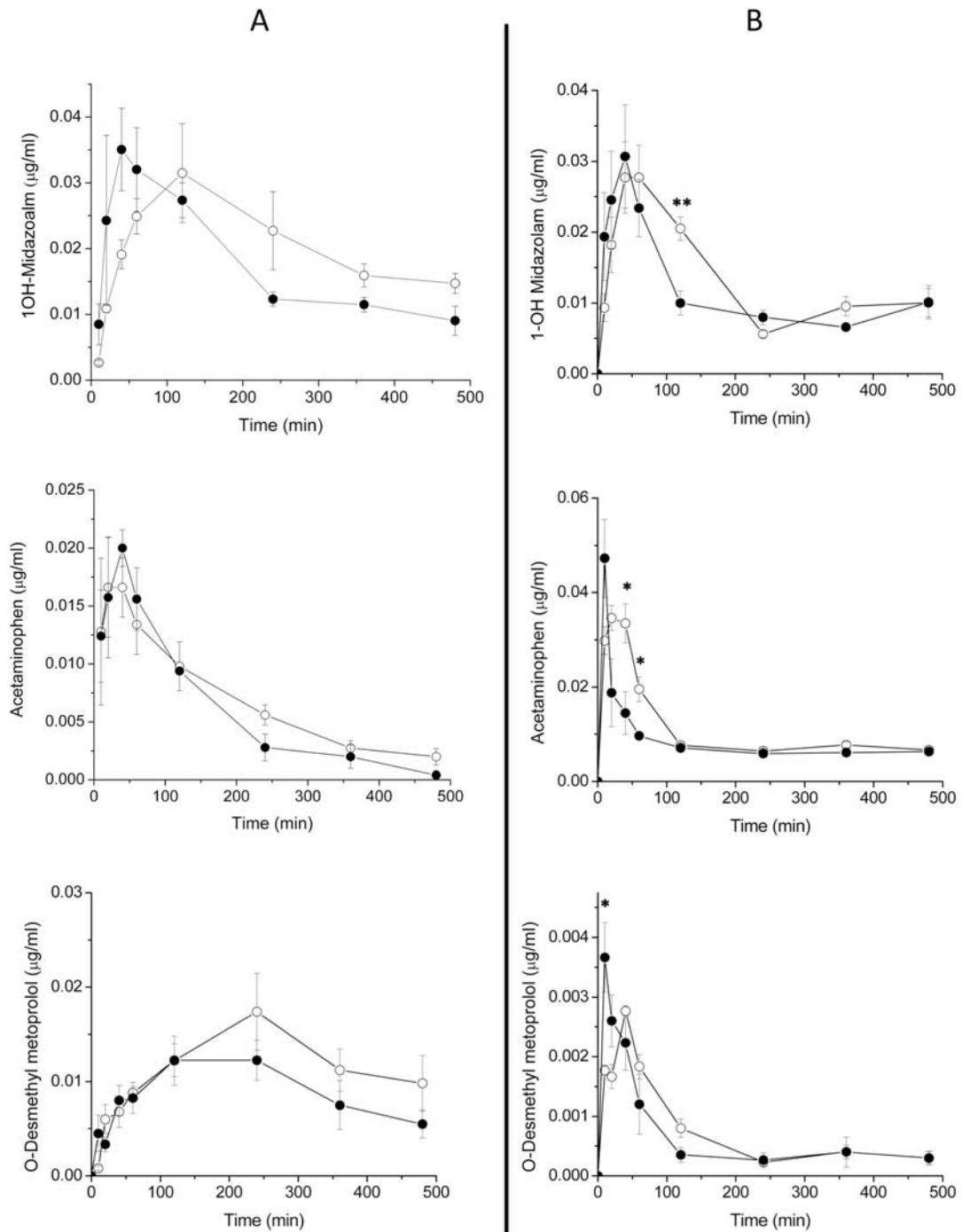
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table 1: Modes of detection and parameters used to quantify the different products formed during microsomal incubations with the LC-MS/MS.

Drug	Ion mode	MRM transitions	Cone voltage (V)	Collision Energy (kV)
4-Hydroxy tolbutamide	- ve	285.1 > 186.1	32	21
Chlorzoxazone (IS)	- ve	168.2 > 131.9	44	25
Acetaminophen	+ ve	180.3 > 110.3	30	30
4-Hydroxy midazolam	+ ve	342.4 > 234.3	30	23
1-Hydroxy midazolam	+ ve	342.4 > 324.3	30	22
O-Desmethyl metoprolol	+ ve	254.1 > 177.2 254.1 > 72.0	35	20
α-Hydroxy metoprolol	+ ve	284.1 > 73.9 284.1 > 116	35	21
Caffeine (IS)	+ ve	195 > 138	35	25
Phenacetin (IS)	+ ve	180.3 > 110.3	30	30
Resorpine	+ ve	607.1 > 137	70	46

Supplementary Table 2: Modes of detection and parameters used for each individual drug measured by LC-MS/MS in plasma samples.

Drug	Ion mode	MRM transitions	Cone voltage (V)	Collision energy (kV)
Tolbutamide	- ve	269.1 > 170.2	30	20
Chlorzoxazone	- ve	168.1 > 131.9	44	25
Resorpine (IS)	- ve	607.1 > 137	70	46
Phenacetin	+ ve	180.3 > 110.3	30	30
Midazolam	+ ve	326.4 > 291.4	30	27
Metoprolol	+ ve	268 > 133	30	26
Caffeine (IS)	+ ve	195 > 138	35	25

Supplementary Table 3: NADPH and NADH dependent P450 activities of wild-type and cytochrome *b*₅ hepatic null microsomes towards a range of P450 model substrates

Assays were performed in triplicate with liver microsomes at substrate concentrations approximately 5 times K_m and turnover rates calculated by reference to authentic standards.

		NADPH				NADH		
Substrate	Metabolite	WT rate (pmol/min/pmol)	HBN rate (pmol/min/pmol)	p value		WT rate (pmol/min/pmol)	HBN rate (pmol/min/pmol)	p value
BFC	HFC	362 ± 120	68 ± 33	0.0002		106 ± 101	0.7 ± 1.7	<0.0001
EFC	HFC	464 ± 165	47 ± 30	<0.0001		88 ± 17	0.7 ± 1.0	<0.0001
MFC	HFC	591 ± 183	64 ± 51	<0.0001		72 ± 18	0.2 ± 0.6	<0.0001
ER	Resorufin	27 ± 5	20 ± 4	0.02		14 ± 3	10 ± 2	0.02
BR	Resorufin	22 ± 10	8 ± 2	0.007		0.9 ± 0.8	1.4 ± 1.0	0.35
Bufuralol	1'-OH Bufuralol	670 ± 189	292 ± 26	0.0007		254 ± 76	72 ± 27	0.002

Supplementary Table 4: Summary of pharmacokinetic data on drugs administered intravenously to HBN mice and wild-type controls.

Adult male HBN and WT mice (n =4) were administered a drug cocktail intravenously; serial blood samples were collected over a 8 hour period and analysed for plasma drug concentrations by LC-MS/MS and key pharmacokinetic parameters calculated.

Drug	PK parameters	WT	HBN	p-value
Midazolam	AUC (0→∞) (min*µg/ml)	244 ± 63	224 ± 43	0.81
	AUC (0→480) (min*µg/ml)	241 ± 63	149 ± 16	0.26
	Clearance (ml/min/kg)	28 ± 8	24 ± 5	0.71
	Terminal half-life (min)	83 ± 15	330 ± 132	0.03
Phenacetin	AUC (0→∞) (min*µg/ml)	522 ± 110	617 ± 85	0.52
	AUC (0→480) (min*µg/ml)	520 ± 110	524 ± 22	0.97
	Clearance (ml/min/kg)	12 ± 3	8.4 ± 1.1	0.34
	Terminal half-life (min)	53 ± 11	168 ± 56	0.02
Metoprolol	AUC (0→∞) (min*µg/ml)	76 ± 19	89 ± 14	0.61

	AUC (0→480) (min*µg/ml)	75 ± 19	64 ± 5	0.66
	Clearance (ml/min/kg)	36 ± 10	24 ± 4.4	0.35
	Terminal half-life (min)	97 ± 14	261 ± 65	0.10
Chlorzoxazone	AUC (0→∞) (min*µg/ml)	432 ± 74	663 ± 127	0.10
	AUC (0→480) (min*µg/ml)	420 ± 75	481 ± 39	0.52
	Clearance (ml/min/kg)	13 ± 2	8.2 ± 1.8	0.13
	Terminal half-life (min)	92 ± 19	258 ± 76	0.02
Tolbutamide	AUC (0→∞) (min*µg/ml)	429 ± 114	672 ± 51	0.06
	AUC (0→480) (min*µg/ml)	367 ± 88	440 ± 33	0.50
	Clearance (ml/min/kg)	14 ± 2	7.5 ± 0.6	0.06
	Terminal half-life (min)	197 ± 37	321 ± 25	0.03

Supplementary Table 5: Summary of pharmacokinetic data on drugs administered orally to HBN and wild-type controls.

Adult male HBN and WT mice (n =4) were administered a drug cocktail orally; serial blood samples were collected over a 8 hour period and analysed for plasma drug concentrations by LC-MS/MS and key pharmacokinetic parameters calculated. For the single dose tolbutamide, n = 2 for WT mice and n= 4 for HBN, therefore precluding statistical analysis of the data.

Drug	PK parameter	WT	HBN	p-value
Midazolam	AUC (0→∞) (min*µg/ml)	34 ± 3	124 ± 7	<0.001
	AUC (0→360) (min*µg/ml)	30 ± 1	120 ± 6	<0.001
	C _{max} (µg/ml)	0.40 ± 0.03	1 ± 0.03	<0.001
	Clearance (ml/min/kg)	149 ± 12	41 ± 2	<0.001
	Terminal half-life (min)	167 ± 58	70 ± 8	0.17
Phenacetin	AUC (0→∞) (min*µg/ml)	2.9 ± 0.4	4.9 ± 0.4	0.03

	AUC (0→240) (min*μg/ml)	1.5 ± 0.1	1.8 ± 0.1	0.06
	C _{max} (μg/ml)	0.06 ± 0.01	0.09 ± 0.02	0.02
	Clearance (ml/min/kg)	688 ± 30	550 ± 38	0.05
	Terminal half-life (min)	308 ± 44	279 ± 11	0.55
Metoprolol	AUC (0→∞) (min*μg/ml)	0.20 ± 0.07	0.22 ± 0.04	0.80
	AUC (0→240) (min*μg/ml)	0.11 ± 0.02	0.16 ± 0.03	0.23
	C _{max} (ng/ml)	1.1 ± 0.1	1.5 ± 0.2	0.09
	Clearance (L/min/kg)	13 ± 4	9.5 ± 1.6	0.51
	Terminal half-life (min)	168 ± 51	130 ± 8	0.50
Chlorzoxazone	AUC (0→∞) (min*μg/ml)	16 ± 2	13 ± 3	0.44
	AUC (0→360) (min*μg/ml)	15 ± 3	13 ± 3	0.57

	C_{\max} (µg/ml)	0.37 ± 0.05	0.20 ± 0.02	0.06
	Clearance (ml/min/kg)	323 ± 42	425 ± 83	0.33
	Terminal half-life (min)	194 ± 31	46 ± 4	0.01
Tolbutamide (cocktail)	AUC (0→∞) (min*µg/ml)	409 ± 58	315 ± 47	0.1
	AUC (0→480) (min*µg/ml)	249 ± 35	126 ± 9	0.02
	C_{\max} (µg/ml)	0.89 ± 0.12	0.34 ± 0.02	0.003
	Clearance (ml/min/kg)	13 ± 2	17 ± 2	0.14
	Terminal half-life (min)	364 ± 33	640 ± 105	0.08
Tolbutamide (single)	AUC (0→∞) (min*µg/ml)	658 ± 269	907 ± 146	-
	AUC (0→480) (min*µg/ml)	344 ± 5	587 ± 47	-

	C_{\max} (µg/ml)	1.4 ± 0.1	2.0 ± 0.2	-
	Clearance (ml/min/kg)	9.1 ± 3.7	6.1 ± 1.2	-
	Terminal half-life (min)	408 ± 249	286 ± 50	-